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Characterisation of chlorinated, brominated and mixed halogenated dioxins, furans and biphenyls as potent and as partial agonists of the Aryl hydrocarbon receptor

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ABSTRACT

The Aryl hydrocarbon receptor (AhR) binds a variety of chlorinated and brominated dioxins, furans and biphenyls. Mixed halogenated variants have been recently identified in food at significant levels but full characterisation requires potency data in order to gauge their impact on risk assessment. Rat H4IIE and human MCF-7 cells were treated with various mixed halogenated ligands. Antagonist properties were measured by treating cells with various concentrations of TCDD in the presence of EC_{25} of the putative antagonist. Measurement of CYP1A1 RNA was used to quantify the potency of agonism and antagonism. The PXDDs were found to be slightly less potent than the corresponding fully chlorinated congeners with the exception of 2-B,3,7,8-TriCDD which was 2-fold more potent than TCDD. PXDFs and non-ortho-PXBs were found to be more potent than their chlorinated congeners whilst several mono-*ortho*-substituted PXBs were shown to have partial agonistic properties. REPs were produced for a range of mixed halogenated AhR-activating ligands providing a more accurate estimation of potency for risk assessment. Several environmentally abundant biphenyls were shown to be antagonists and reduce the ability of TCDD to induce CYP1A1. The demonstration of antagonism for AhR ligands represents a challenge for existing REP risk assessment schemes for AhR ligands.

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1. Introduction

The Aryl hydrocarbon receptor (AhR) binds a wide range of structurally diverse compounds such as dibenzo-*p*-dioxins, dibenzofurans and biphenyls (Denison and Nagy, 2003). These AhR ligands, whether chlorinated, brominated or a mixture of both, all undergo the same mechanism of action by activating the AhR. Activation of the AhR leads to the induction of a battery of xenobiotic enzymes such as cytochrome P4501A1 (CYP1A1; Behnisch et al., 2001; Schmidt et al., 1996; Vanden Heuvel et al., 1994). Thus measurement of CYP1A1 expression is a widely known and accepted method of determining AhR activation. Activation of the AhR is generally required to instigate the toxic effects of AhR agonists, as demonstrated in AhR-null mice, which are resistant to the acute toxicity of TCDD (Gonzalez and Fernandez-Salguero, 1998; Lin et al., 2002; Stohs and Hassoun, 2011). A more detailed review of AhR activation can be found elsewhere (Denison et al., 2011).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most potent and certainly the best characterised of the AhR ligands. TCDD is used as a reference compound when estimating the relative estimated potency (REP), a measure of toxic strength in comparison to TCDD, for other AhR agonists. The most potent polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzonfuran (PCDFs) and polychlorinated biphenyls (PCBs) feature in the World Health Organisation's (WHO) evaluation of toxic equivalency factors (TEFs) of dioxin-like compounds (European Commission, 2011; Haws et al., 2006; van den Berg et al., 2006). The TEF values established by the WHO consortium were based on relative effect potency (REP) values derived from a meta-analysis of previous potency data (Haws et al., 2006). The TEFs are used to estimate the total toxic equivalency (TEQ) of a mixture of compounds in an additive fashion by combining the TEF of each compound with the concentration of the compound within the mixture (van den Berg et al., 2006). The dataset contains the potent but less abundant TCDD (TEF = 1) and PCB 126 (TEF = 0.1) and the more environmentally abundant but considerably less potent mono-*ortho*-substituted PCBs (TEF = 0.00003).

A great deal of research has been conducted on chlorinated dibenzo-*p*-dioxins, dibenzofurans (PCDD/Fs) and biphenyls (PCBs), and a more limited amount on their brominated analogues (PBDD/Fs, PBBs), with regard to their toxicity and occurrence in the environment and in food. However little is known about mixed (chlorinated and brominated) halogenated dibenzo-*p*-dioxins (PXDDs), dibenzofurans (PXDFs)

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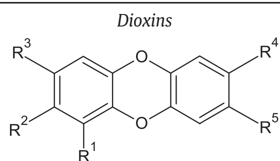
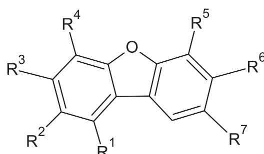
Table 1
Structures of the mixed halogenated dibenzodioxins (PXDDs), dibenzofurans (PXDFs) and biphenyls (PXBs) used in this study.

Dioxins



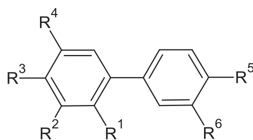
2-B-7,8-DiCDD – R^1 = H; R^2 = Br; R^3 = H; R^4 = Cl; R^5 = Cl
2,3,7-TriBDD – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Br; R^5 = H
2,3,7,8-TetraCDD – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Cl
2-B,3,7,8-TriCDD – R^1 = H; R^2 = Br; R^3 = Cl; R^4 = Cl; R^5 = Cl
2,3-DiB,7,8-DiCDD – R^1 = H; R^2 = Br; R^3 = Br; R^4 = Cl; R^5 = Cl
1-B,2,3,7,8-TetraCDD – R^1 = Br; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Cl
2-B,1,3,7,8-TetraCDD – R^1 = Cl; R^2 = Br; R^3 = Cl; R^4 = Cl; R^5 = Cl

Furans



2,7,8-TriBDF – R^1 = H; R^2 = Br; R^3 = H; R^4 = H; R^5 = H; R^6 = Br; R^7 = Br
2-B,7,8-DiCDF – R^1 = H; R^2 = Br; R^3 = H; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl
2,3,7,8-TetraCDF – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl
3-B,2,7,8-TriCDF – R^1 = H; R^2 = Cl; R^3 = Br; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl
2,3-DiB,7,8-DiCDF – R^1 = H; R^2 = Br; R^3 = Br; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl
2-B,6,7,8-TriCDF – R^1 = H; R^2 = Br; R^3 = H; R^4 = H; R^5 = Cl; R^6 = Cl; R^7 = Cl
2,3,4,7,8-PentaCDF – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = H; R^6 = Cl; R^7 = Cl
4-B,2,3,7,8-TetraCDF – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Br; R^5 = H; R^6 = Cl; R^7 = Cl
1-B,2,3,7,8-TetraCDF – R^1 = Br; R^2 = Cl; R^3 = Cl; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl
1,3,DiB,2,7,8-TriCDF – R^1 = Br; R^2 = Cl; R^3 = Br; R^4 = H; R^5 = H; R^6 = Cl; R^7 = Cl

Biphenyls



3,3′,4,4′,5-PentaCB (PCB 126) – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Cl; R^6 = Cl
4′-B,3,3′,4,5-TetraCB (PXB 126B) – R^1 = Hl; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Br; R^6 = Cl
3′,4′-DiB,3,4,5-TriCB (PXB 126H) – R^1 = H; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Br; R^6 = Br
3′,4′,5-TriB,3,4-DiCB (PXB 126V) – R^1 = H; R^2 = Br; R^3 = Cl; R^4 = Br; R^5 = Br; R^6 = Br
3,3′,4,4′,5-PentaBB (PBB 126) – R^1 = H; R^2 = Br; R^3 = Br; R^4 = Br; R^5 = Br; R^6 = Br
2,3,3′,4,4′-PentaCB (PCB 105) – R^1 = Cl; R^2 = H; R^3 = Cl; R^4 = Cl; R^5 = Cl; R^6 = Cl
4′-B,2,3,3′,4-TetraCB (PXB 105) – R^1 = Cl; R^2 = H; R^3 = Cl; R^4 = Cl; R^5 = Br; R^6 = Cl
2,3′,4,4′,5-PentaCB (PCB 118) – R^1 = Cl; R^2 = Cl; R^3 = Cl; R^4 = H; R^5 = Cl; R^6 = Cl
4′-B,2,3′,4,5-TetraCB (PXB 118) – R^1 = Cl; R^2 = Cl; R^3 = Cl; R^4 = H; R^5 = Br; R^6 = Cl
2,3,3′,4,4′,5-HexaCB (PCB 156) – R^1 = Cl; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Cl; R^6 = Cl
4′-B,2,3,3′,4,5-PentaCB (PXB 156) – R^1 = Cl; R^2 = Cl; R^3 = Cl; R^4 = Cl; R^5 = Br; R^6 = Cl

and biphenyls (PXBs) which have recently been found at comparable levels in various food products (Fernandes et al., 2011, 2014). Unintentional formation of these compounds occurs at equivalent levels to chlorinated and brominated congeners (Du et al., 2010) through combustion processes. Currently PBDD/Fs and PBBs are allocated the same REP as their chlorinated counterparts (Fernandes et al., 2008; van den Berg et al., 2013), however, the limited potency work that has been conducted on PXDD/Fs and PXBs has shown some of them to be more potent and hence may have a bigger impact on the TEQ of a mixture (Behnisch et al., 2003; Olsman et al., 2007; Samara et al., 2009).

Another issue regarding the TEQ is the putative effect of antagonists in the estimation of the potency of a mixture. Research in vitro on some mono-*ortho*-substituted PCBs has suggested that several of the congeners have both agonistic and antagonistic properties (Clemons et al., 1998; Hestermann et al., 2000). These antagonist effects may reduce the overall potency of a mixture by inhibiting more potent 'pure' agonists from inducing CYP1A1. Three of the most abundant mono-*ortho*-substituted PCBs (PCB 105, PCB 118 and PCB 156), have been found in significant quantities in environmental studies (Ahlborg et al., 1992; Fernandes et al., 2004, 2008; Larebeke et al., 2001; Kalantzi et al., 2004; Polder et al., 2008; Safe, 1990, 1994) showing the potential to have a large impact on the final TEQ.

Here, we calculate the agonist potency of a range of mixed halogenated PXDDs, PXDFs and PXBs which are currently not included in the WHO's TEF list. Secondly, we evaluate the putative antagonistic effects of several of the most abundant mono-*ortho*-substituted PCBs and PXBs on the TEQ using a novel method of quantifying the antagonist effect of various compounds on AhR activation.

2. Materials and methods

2.1. Materials

Dibenzo-*p*-dioxin, dibenzofuran and biphenyl standards were obtained either from Wellington Laboratories Inc. Ontario, Canada or from Cambridge Isotope Labs, Mass. USA. Where required, the standards were solvent exchanged to DMSO and the concentrations verified using Gas chromatography/Mass spectroscopy (GC/MS). The chemical names for the 3,3′,4,4′,5-substituted-mixed halogenated biphenyls have been published previously (Falandysz et al., 2012). A trace (<0.01%) of either nonane, toluene or iso-octane may be present but were all shown to have no effect on cell growth or AhR activation up to concentrations of 0.1%. A summary of the structures of the compounds can be found in Table 1. Dilution of TCDD was done in DMSO to 10 μM which was stored at −20 °C. A 10 mM mono-*ortho*-substituted PCB top stock was made by dissolving in DMSO and stored at −20 °C. The remaining compounds were dissolved in DMSO up to a concentration of 100 μM or 1 mM. Once constituted, all further dilutions were made using conditioned medium, giving a final DMSO concentration of <0.2%.

2.2. Confirmation of concentration and absence of contamination

High resolution gas chromatography coupled to high resolution mass spectrometry (HRGC/HRMS) was used to verify the concentrations used and confirm the absence of contaminants in stock PCB, PXDD/F and PXB experimental solutions (data not shown). The mass spectrometer (Autospec Premier Waters) was operated in electron ionisation (EI) mode at a mass resolution of 10000 for PCBs/PCDD/Fs and 13,000–15,000 (at 10% peak height) for PXBs, PXDD/Fs with the mass axis calibrated within a window of $250ppm_{mass}$ prior to measurement. An acceleration voltage of 7 kV was used in conjunction with an electron energy of 32–37 eV and a trap current of 450 μA. The two most intense ions that did not suffer from chemical interference, in the molecular ion cluster for each analyte, were targeted and were separated into discrete groups based on the molecular mass range and chromatographic retention. The full validated methodology has been described earlier (Fernandes et al., 2004, PCBs; Fernandes et al., 2011, PXDD/Fs).

2.3. Cell culture

The human MCF-7 cells were a kind gift from Dr Tracey Bradshaw (Centre for Biomolecular Science, University of Nottingham, UK) and the rat H4IIE-C3 cells (#85061112) were purchased from the ECACC. The two cell lines were maintained in minimum essential medium (MEM; Sigma #M2279) with the addition of 10% foetal bovine serum (Sigma #F7524), antibiotics (Sigma #G1146) and 1% non-essential amino acids (Sigma #M7145). Cells were passaged every 3 days and incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Agonism measurement

Rat H4IIE and human MCF-7 cells were treated with test compounds to determine their potency. To measure the agonistic properties of the compounds, cells were seeded in 96-well plates (cell density of 1.5 $\times 10^5$ cells/well) and treated with the compounds (100 fM 10 μM) for 4 h. Conditioned medium (used medium from untreated cells) was used to prevent contamination from AhR agonists in the fresh medium (Adachi et al., 2001). A vehicle only control (no activation) and a 10 nM TCDD only control (maximum activation) were run alongside the concentration–response (C/R) curves to be used for normalisation purposes. Three biological replicates (separate cell samples in individual wells) were run for each concentration point. The cells were removed from the plate using 60 μl trypsin (Sigma #T4174) and frozen at −20 °C. The RNA was then purified using Absolutely RNA® Miniprep Kit (Stratagene #400800) as per instructions for small sample sizes. cDNA synthesis was completed using a High capacity RNA-to-cDNA kit (Applied Biosystems #4387406), samples were incubated for 60 min at 37 °C followed by 5 min at 95 °C using an Eppendorf thermocycler (Germany). Alongside the samples, a no reverse-transcriptase (No RT) and a No RNA control were prepared for control purposes. Samples were stored at −20 °C.

2.5. Antagonism measurement

In addition to the methodology discussed for agonism, to measure the antagonistic properties, cells were treated with TCDD (100 fM 10 nM) in the presence of the set concentration (~EC_{25}) of the test compound as identified in the agonism experiments. Extraction of RNA and measurement of CYP1A1 RNA was as described for the agonism experiments. The basis for studying the effect of antagonist on potency of TCDD for inducing CYP1A1 RNA has been described previously (Bazzi et al., 2009; Wall et al., 2012a, 2012b).

2.6. qRT-PCR measurement of CYP1A1 RNA

RNA measurement used quantitative real-time PCR to provide a real-time view of RNA levels. CYP1A1 was measured and normalised against two reference genes, β-actin and AhR.

Only a single endpoint was used in this study, a common method used in the literature for REP calculations. A complete master mix was prepared containing 20 μl Taqman® gene expression master mix (Applied Biosystems #4369016), CYP1A1, β-actin and AhR primers (10 μM) and probes (5 μM), 150 ng of cDNA and diethylpyrocarbonate (DEPC) treated water (made up to 40 μl). The nucleotide sequences for the primers and probes have been published previously (Wall et al., 2012b). Previous work has shown that these primers and probes can be run in the same reaction without interference (Bazzi et al., 2009; Bell et al., 2007).

The samples were run as two technical replicates (20 μl each) on a 96-well plate. The RNA levels were detected using an Applied Biosystems 7500 Fast RT-PCR machine with the following protocol: 1 cycle (2 min at 50 °C; 10 min at 95 °C), 40 cycles (20 s at 95 °C; 90 s at 58 °C). Three controls, to check for contamination, were run alongside each curve; no template control (NTC), No RT and No RNA. The RNA levels were measured by their C_t values (the cycle at which the fluorescence passes a set threshold and is distinguishable from the background noise) and analysed using qBasePlus v1.3 (Biogazelle). The C_t or copy number values for CYP1A1 were normalised against the values obtained for β-actin and AhR, and converted into calibrated normalised relative quantities (CNRQ; Hellemans et al., 2007; Vandesompele et al., 2002). These values were then further normalised against CYP1A1 RNA levels from a contemporaneous culture exposed to 10 nM TCDD which was defined as 100% or maximal response. The data was then plotted as concentration vs. normalised CYP1A1 RNA (% of maximal response) using GraphPad Prism 5 which also calculated the EC_{50}s and the 95% confidence intervals (95% CI). It was assumed that all the compounds would reach 100% response therefore the agonist curves were fitted to account for this. The C/R curve was corrected for all the antagonist studies. The EC_{50} was calculated as the halfway point between the ~25% background, due to the antagonist, and the 100% maximal induction. An unpaired t-test was used to test whether any difference in the EC_{50}s derived from the antagonism curves was statistically significant.

3. Results

3.1. Agonistic properties of mixed halogenated congeners

To ascertain the potency of the selected mixed halogenated compounds and to establish whether they are as potent than their chlorinated congeners, this study tested the ability of these compounds to activate the AhR in H4IIE rat cells, an established methodology for examining potency (Bazzi et al., 2009; Behnisch et al., 2003; Olsman et al., 2007; Samara et al., 2009). A key parameter of the experimental design was the treatment of rat H4IIE cells with the compounds for 4 h, prior to quantitation of CYP1A1 RNA using qRT-PCR (Fig. 1A–E), since longer treatment periods increase the risk of metabolism of the chemicals and may result in altered potency estimates (Supplemental Fig. 1; Bazzi et al., 2009). All of the PXDDs tested were within 20-fold less potent than TCDD with the exception of 2-B,3,7,8-TriCDD which was 2-3-fold more potent (Fig. 1A). The PXDFs were also within 20-fold less potent than TCDD with the exception of 2-B,7,8-DiCDF which was shown to be ~2000-fold less potent than TCDD (Fig. 1B and C). The non-*ortho*-substituted PXBs tested were within 10-fold less potent than TCDD (Fig. 1D) and the mono-*ortho*-substituted PXBs were shown to be at least 5000-fold less potent than TCDD (PCB 156 and PXB 156; Fig. 1E). Interestingly, the PXDFs and non-*ortho*-substituted PXBs were all more potent than their fully chlorinated counterparts. The results are summarised in Table 2.

Previous work has shown that certain compounds can have different potencies in different species, with TCDD ~10-fold less potent in human MCF-7 cells than in rat H4IIE cells (Wall et al., 2012a, 2012b), in agreement with other literature (Budinsky et al., 2010; Xu et al., 2000). To test if there is lower potency in human for a variety of PCDDs and PXBs, and to relate the data gathered in rats to a human model, several of the compounds were tested in human MCF-7 cells so that a direct comparison of potency could be established (Fig. 1F). TCDD and 2-B,3,7,8-TriCDD gave EC_{50}s in rat H4IIE cells (Fig. 1A) of 47.6 pM (95% CI = 36.4 pM–62.2 pM) and 23.7 pM (95% CI = 15.4 pM–36.6 pM) respectively, whereas in the human MCF-7 cells, which were treated under the same experimental conditions, the EC_{50}s were 465 pM (95% CI = 341 pM–633 pM) and 187 pM (95% CI = 111 pM–319 pM) for TCDD and 2-B,3,7,8-TriCDD, respectively. Thus, TCDD was 10-fold more potent in rat H4IIE cells than in human MCF-7 ($p < 0.001$) whereas 2-B,3,7,8-TriCDD was 8-fold more potent in rat H4IIE ($p < 0.001$). Consequently, most of the compounds were found to be 6- to 30-fold less potent in human MCF-7 compared to rat H4IIE cells, except for two compounds where it was not possible to reliably determine the EC_{50} in human cells. This shows that the lower potency seen in human MCF7 cells compared with rat H4IIE cells is a consistent finding for multiple poly-halogenated dioxins and biphenyls.

3.2. Antagonist effects of mono-ortho-PCBs

The TEQ system assumes that the agonist properties of each compound can be added together in a mixture to give a cumulative estimate of potency. However, this system does not take into account antagonists and their potential to reduce the overall potency by inhibiting other more potent compounds. Therefore we set out to establish if antagonism is an important consideration for the dioxin and biphenyl series by quantitatively measuring the putative effects of antagonists in a mixture with pure agonists. Several mono-*ortho*-

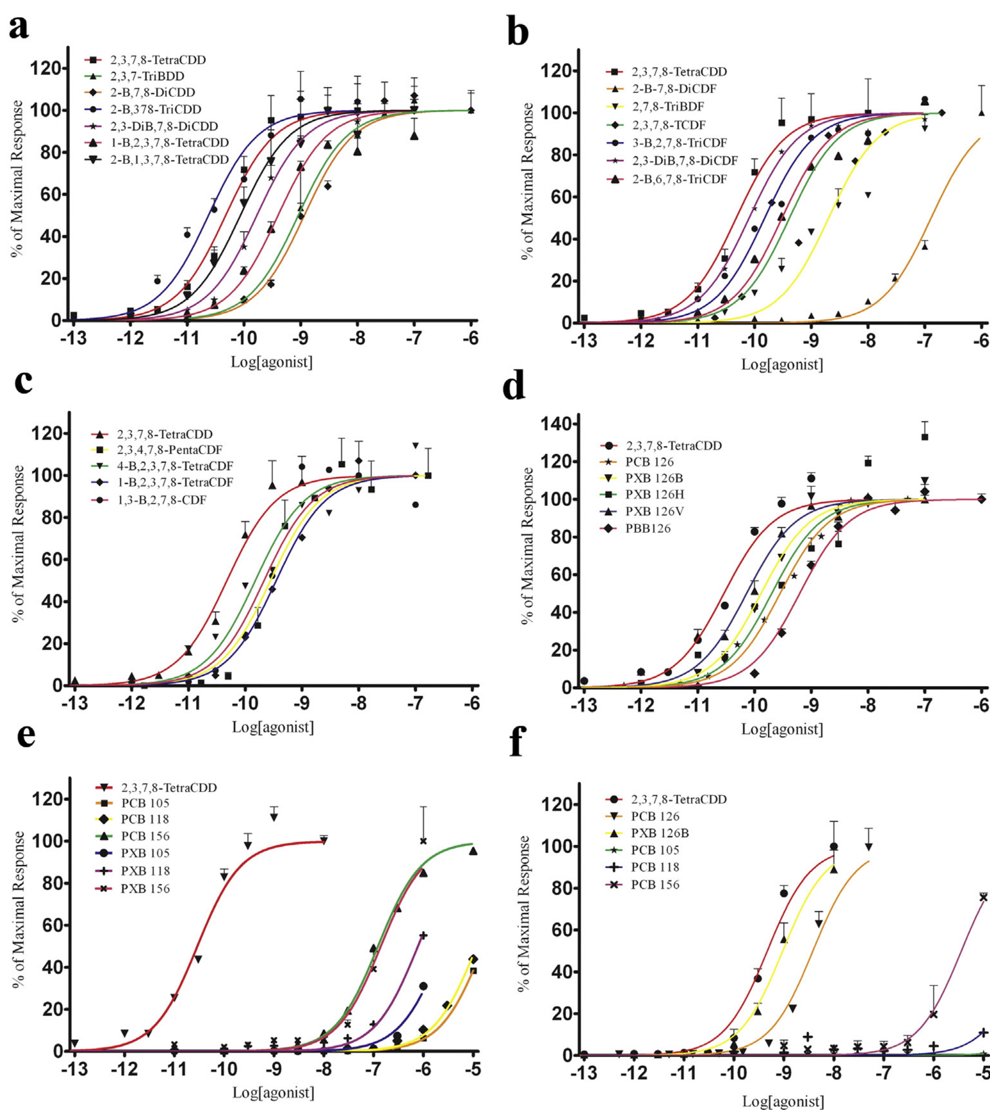


Fig. 1. Potency comparison of PXDD, PXDF and PXB congeners — Concentration–response relationships for rat H4IIE cells treated for 4 h with (A) PXDDs, (B) PXDFs (Tri and Tetra substituted), (C) PXDFs (Penta substituted), (D) Non-*ortho*-substituted PXBs, (E) Mono-*ortho*-substituted PXBs and (F) human MCF-7 cells treated with a variety of PXBs. qRT-PCR was used to measure the level of induction of CYP1A1 and compared against control genes, β-actin and AhR. qBasePlus was used to normalise the data which was plotted using 10 nM TCDD only control as the maximal response (100%). Each point consists of three biological replicates ± SEM.

PCBs and PXBs, which are known to occur in food and the environment, were tested in combination with TCDD using a novel method of measuring antagonism developed previously (Bazzi et al., 2009). Rat H4IIE cells were treated for 4 h with various concentrations of TCDD in the presence of an empirically-determined concentration of the PXB, the concentration giving a 25% of maximal agonist response (EC_{25}) (PCB 105, 3 μM; PCB 118, 3 μM; PCB 156, 30 nM; Fig. 1E). PCB 105 and PCB 118 increased the EC_{50} of TCDD for inducing CYP1A1 by 30-fold and 10-fold, respectively ($p < 0.001$) and in combination with the agonist data (Fig. 1E), were both shown to be partial agonists (both agonist and antagonist properties) in rat H4IIE cells. PCB156 was identified as a pure agonist. The effect of a chlorine atom substituted for a bromine atom (position R^5; Table 1) was then investigated in rat H4IIE cells. PXB 105, PXB 118 and PXB 156 all led to an increase of 200-fold ($p < 0.001$), 4-fold ($p < 0.01$) and 100-fold ($p < 0.001$), respectively, in the EC_{50} of TCDD for inducing CYP1A1 RNA (Fig. 2B). In combination with agonist data (Fig. 1E), all three compounds have agonistic and antagonistic behaviour, and are partial agonists. Finally in rat H4IIE cells, the antagonistic properties of PCB 126, TCDF and PeCDF were tested (Fig. 2C). The compounds had no effect on the EC_{50} of TCDD for inducing CYP1A1 RNA. All of the EC_{50}s derived from the antagonist experiments are summarised in Table 3 and show that the compounds increased the EC_{50} of TCDD by up to 200-fold compared with TCDD alone.

Although it is known that there can be species-specific agonism of the AhR, it is not known if there is species-specific antagonism of the AhR. The antagonist effects of some of these compounds were therefore tested in a human MCF-7 cells to see if they have the similar effect of reducing the ability of more potent compounds to induce CYP1A1. Human MCF-7 cells were treated with either the EC_{25} based on Fig. 1F (300 nM; PCB 156) or when no response was detected in human cells, the highest tested concentration based on the solubility of the compounds (10 μM; PCB 105 and PCB 118). A concentration of 10 μM PCB 105 or PCB 118 had very little agonistic effect in human cells (Fig. 1F) but both produced a significant antagonist effect on the TCDD response with PCB 105 and PCB118 having a 35-fold and 25-fold reduction in mixture potency, respectively ($p < 0.001$; Fig. 2D). PCB 156 also produced an antagonist effect in human MCF-7 cells (3-fold reduction; $p < 0.01$) as well as being a poor agonist. From this combined data it is possible to conclude that PCB 105 is a partial agonist in rat H4IIE cells and an antagonist in human MCF-7 at the concentrations tested, PCB 118 is a partial agonist

Table 2
Summary of REP values calculated in this study and comparison with the literature.

Compound	Rat		Human		2005 WHO TEF	Behnisch et al. (2003)	Olsman et al. (2007)	Samara et al. (2009)
	EC_{50} (95% CI)	REP	EC_{50} (95% CI)	REP				
2,3,7-TriBDD	904 pM (710 pM–1.15 nM)	0.052	–	–	–	0.033	0.081	0.0006
2-B-7,8-DiCDD	1.16 nM (813 pM–1.64 nM)	0.041	–	–	–	–	0.061	–
2,3,7,8-TetraCDD	47.6 pM (36.4 pM–62.2 pM)	1[a]	465 pM (341 pM–633 pM)	1	1	1	1	1
2-B,3,7,8-TriCDD	23.7 pM (15.4 pM–36.6 pM)	2.01	187 pM (111 pM–317 pM)	2.4847	–	0.67	1.93	0.72
2,3-DiB-7,8-DiCDD	168 pM (138 pM–206 pM)	0.28	–	–	–	0.86	1.00	0.43
1-B,2,3,7,8-TetraCDD	398 pM (264 pM–599 pM)	0.12	–	–	–	0.28	–	–
2-B,1,3,7,8-TetraCDD	86.4 pM (65.3 pM–114 pM)	0.55	–	–	–	0.37	1.52	–
2,7,8-TriBDF	2.02 nM (1.10 nM–3.71 nM)	0.024	–	–	–	–	0.00049	–
2-B-7,8-DiCDF	129 nM (90.1 nM–184 nM)	0.00037	–	–	–	–	0.000037	–
2,3,7,8-TetraCDF	413 pM (196 pM–869 pM)	0.12	–	–	0.1	0.32	–	0.07
3-B,2,7,8-TriCDF	151 pM (106 pM–214 pM)	0.32	–	–	–	0.74	–	0.38
2,3-DiB,7,8-DiCDF	80.3 pM (65.0 pM–99.1 pM)	0.59	–	–	–	–	–	–
2-B,6,7,8-TriCDF	305 pM (214 pM–434 pM)	0.16	–	–	–	–	0.00066	–
2,3,4,7,8-PentaCDF	278 pM (179 pM–433 pM)	0.17	–	–	0.3	0.5	–	0.46
4-B,2,3,7,8-TetraCDF	150 pM (86.9 pM–260 pM)	0.32	–	–	–	–	–	–
1-B,2,3,7,8-TetraCDF	351 pM (275 pM–449 pM)	0.14	–	–	–	–	–	–
1,3,DiB,2,7,8-TriCDF	214 pM (119 pM–386 pM)	0.22	–	–	–	–	–	–
2,3,7,8-TetraCDD	28.9 pM (19.9 pM–41.9 pM)	1[b]	–	–	1	1	1	1
3,3′,4,4′,5-PentaCB	281 pM (225 pM–352 pM)	0.10	3.81 nM (2.89 nM–5.02 nM)	0.1221	0.1	0.067	–	–
4′-B,3,3′,4,5-TetraCB	130 pM (92.2 pM–183 pM)	0.22	947 pM (807 pM–1.11 nM)	0.4908	–	–	–	–
3′,4′-DiB,3,4,5-TriCB	200 pM (78.2 pM–513 pM)	0.14	–	–	–	–	–	–
3′,4′,5-TriB,3,4-DiCB	72.2 pM (48.4 pM–108 pM)	0.40	–	–	–	–	–	–
3,3′,4,4,5-PentaBB	622 pM (487 pM–796 pM)	0.046	–	–	–	0.16	–	–
2,3,3′,4,4′-PentaCB	16.0 µM (15.6 µM–16.3 µM)	0.000002	N/A[c]	<0.00005	0.00003	0.000012	–	–
4′-B,2,3,3′,4-TetraCB	2.46 µM (2.10 µM–2.90 µM)	0.00001	–	–	–	–	–	–
2,3′,4,4′,5-PentaCB	11.8 µM (10.5 µM–13.2 µM)	0.000003	N/A[c]	<0.00005	0.00003	0.00001	–	–
4′-B,2,3′,4,5-TetraCB	775 nM (655 nM–917 nM)	0.00003	–	–	–	–	–	–
2,3,3′,4,4′,5-HexCB	122 nM (104 nM–144 nM)	0.0002	3.59 µM (2.81 µM–4.59 µM)	0.0001	0.00003	0.0002	–	–
4′-B,2,3,3′,4,5-PentaCB	139 nM (95.5 nM–203 nM)	0.0002	–	–	–	–	–	–

[a] TCDD value used for dioxin and furan REP estimations.
[b] TCDD value used for PXB REP estimations.
[c] Not enough data to calculate an EC_{50}.

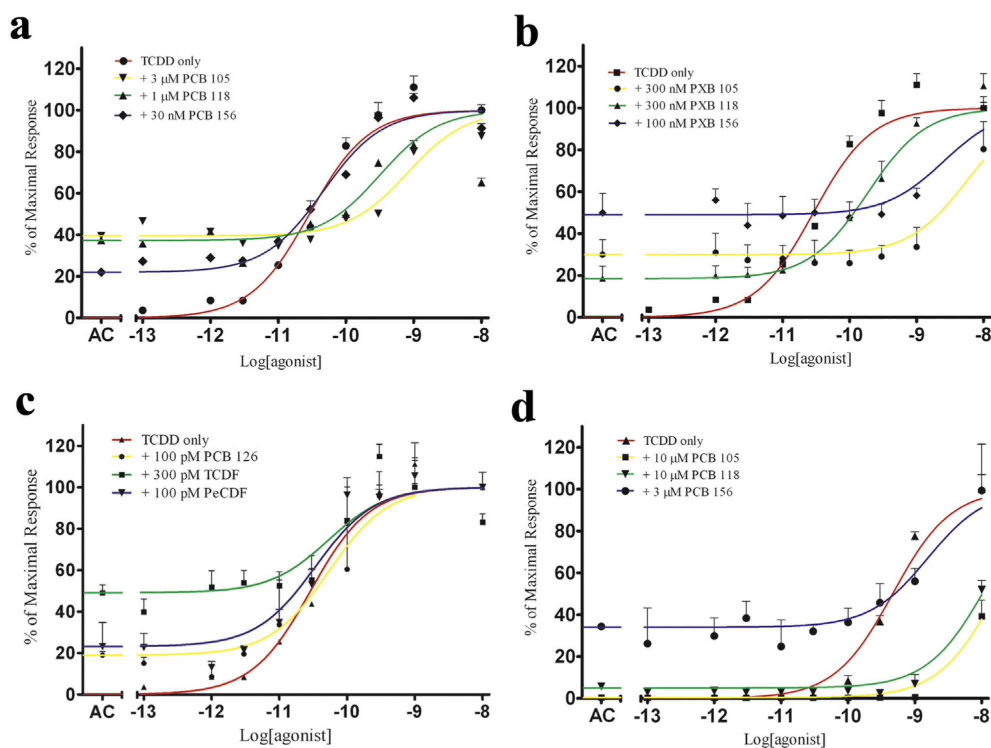


Fig. 2. Antagonistic properties of PXBs and PXDFs — Concentration–response relationships for TCDD in the absence and presence of a set concentration of test compound (EC_{25}) in cells treated for 4 h. Three of the most abundant mono-*ortho*-substituted PCBs were tested in (A) rat H4IIE or (B) human MCF7 cells. Cells were tested with various concentrations of PCB 105, PCB 118 or PCB 156 for 4 h. (C) Mixed halogenated mono-*ortho*-substituted PXBs were tested in rat H4IIE cells. (D) The antagonistic properties of several well characterised furans and a PCB were tested in rat H4IIE cells. qRT-PCR was used to measure the level of induction of CYP1A1 and compared against control genes, β-actin and AhR. qBasePlus was used to normalise the data which was plotted using 10 nM TCDD only control as the maximal response (100%). All of the results were compared with an antagonist only control (AC). Each point consists of three biological replicates ± SEM.

Table 3
Summary of TCDD EC_{50}'s in the absence and presence of selected PXB and PXDF antagonists.

Compound	EC_{50} (95% confidence interval)[a]	Fold decrease
Rat		
TCDD	29 pM (20 pM–42 pM)**	
+ 3 μM PCB 105	810 pM (190 pM–3.4 nM)**	30-fold
+ 1 μM PXB 105	5.6 nM (1.6 nM–19 nM)**	200-fold
+ 3 μM PCB 118	310 pM (75 pM–1.3 nM)**	10-fold
+ 300 nM PXB 118	96 pM (46 pM–200 pM)*	3-fold
+ 30 nM PCB 156	46 pM (25 pM–86 pM)	–
+ 100 nM PXB 156	2.7 nM (170 pM–43 nM)**	100-fold
+ 100 pM PCB 126	54 pM (27 pM–110 pM)	–
+ 300 pM TCDF	53 pM (6.6 pM–430 pM)	–
+ 100 pM PeCDF	31 pM (16 pM–61 pM)	–
Human		
TCDD	470 pM (340 pM–630 pM)**	
+ 10 μM PCB 105[b]	17 nM (14 nM–20 nM)**	35-fold
+ 10 μM PCB 118[b]	11 nM (8.6 nM–14 nM)**	25-fold
+ 300 nM PCB 156	1.6 nM (410 pM–5.9 nM)*	3-fold

[a] Significance indicates that the EC_{50} for TCDD with antagonist is statistically different from TCDD alone in each species *($p < 0.01$) and **($p < 0.001$).
[b] No or limited measurable agonistic effect was detected so the maximum concentration based on the solubility of the compound was used.

in rat and human, and finally PCB 156 was an agonist in rat and a partial agonist in human.

4. Discussion

4.1. Mixed halogenated congeners

One of the main aims of this paper was to characterise the agonist properties of several mixed halogenated AhR ligands based on their ability to induce CYP1A1 RNA. REPs were calculated for most of the compounds tested (Table 2). In rat H4IIE cells, TCDF and PCB 126 both gave REPs of 0.1 and PeCDF gave a REP of 0.2 which corresponds well with the TEFs calculated by the WHO consortium (van den Berg et al., 2006) and shows reproducibility of results compared with those found in the literature. Mason et al. (1987) tested a small selection of mixed halogenated compounds in rat H4IIE cells producing EC_{50}s within 10-fold of this study although they found a much lower value for 2-B,3,7,8-TriCDD than in this or other literature (Mason et al., 1987). REPs identified for mixed halogenated compounds in this paper were compared against values calculated in the literature (Table 2; Behnisch et al., 2003; Olsman et al., 2007; Samara et al., 2009). However, a key element of this experimental method was the robust design for quantification. The use of a 4 h induction period was crucial to minimise the effect of P450 induction and degradation of the substance, thus making it a much better measure of intrinsic agonism. Furthermore the compounds used in this study were fully validated both to confirm concentration and to control for contaminants, allowing more confidence in the data obtained.

All of the REPs calculated for the mixed halogenated compounds tested in this study were within 10-fold of their fully chlorinated counterparts. Table 4 compares the REPs calculated in this paper with the chlorinated congener with the same structure. A few compounds of notable potency were identified including 2-B,3,7,8-TriCDD which was found to be 2- to 2.5-fold more potent than TCDD in both rat and human cell lines. Also identified as very potent AhR agonists were 2-B,1,3,7,8-TetraCDD and 2,3-DiB,7,8-DiCDF, which gave REPs of ~0.6 corresponding well with values found in the literature (Behnisch et al., 2003; Olsman et al., 2007).

The addition of bromine had different effects on each of the three groups of compounds. A bromine substitution on the dibenzo-*p*-dioxin backbone skeleton reduced the potency of the compounds, with the exception of 2-B,3,7,8-TriCDD. In contrast, the substitution of bromine on the furan backbone skeleton increased the potency of the mixed halogenated compounds. One suggestion for this would be that the furan backbone is slightly smaller than the dibenzo-*p*-dioxin backbone skeleton as it only has one oxygen atom thus we propose that the increased size of the bromine atom provides a better fit for the AhR ligand binding domain. This also appears to be the same for the mixed halogenated PXB 126 congeners, with the bromine making the molecule bigger and thus potentially a better fit for the AhR binding domain. Risk assessment of mixtures containing PBB 126 uses the TEF for PCB 126, however, PBB 126 was found to be 3-fold less potent than PCB 126. The mono-*ortho*-substituted PXBs were generally more potent than their chlorinated congeners showing that the increased size of the compound allows it to activate the AhR more effectively. There were significant discrepancies between the REPs calculated for PCB 105, PCB 118 and PCB 156 in this paper and those calculated by the WHO consortium (van den Berg et al., 2006). This work suggests that PCB 105 and PCB 118 were 10-fold less potent than previously estimated whereas PCB 156 was 10-fold more potent. The WHO consortium used a wide range of REPs, derived using multiple endpoints and methodology, to estimate the potency of each compound demonstrating the difficulty in concise TEF calculation (Haws et al., 2006; van den Berg et al., 2006) and thus the importance of using a quantitatively accurate method of measurement. Additionally, differing measurement systems or contaminated stock with more potent ligands may also produce differing potencies (DeVito, 2003), one of the main reasons for verifying that the stocks used in this study were uncontaminated.

Table 4
Estimated REPs compared with TEFs.

Compound	WHO 2005 TEF[a]	Estimated REP from this study[b]	Difference
2,3,7,8-TetraCDD	1	1	=
2-B,3,7,8-TriCDD		3	+ 3-fold
2,3-DiB-7,8-DiCDD		0.3	− 3-fold
1,2,3,7,8-PentaCDD	1		
1-B,2,3,7,8-TetraCDD		0.1	− 10-fold
2-B,1,3,7,8-TetraCDD		0.3	− 3-fold
2,3,7,8-TetraCDF	0.1	0.1	=
3-B,2,7,8-TriCDF		0.1	=
2,3-DiB,7,8-DiCDF		0.3	+ 3-fold
2,3,4,7,8-PentaCDF	0.3	0.3	=
4-B,2,3,7,8-TetraCDF		0.3	=
1,2,3,7,8-PentaCDF	0.03		
1-B,2,3,7,8-TetraCDF		0.1	+ 3-fold
1,3,DiB,2,7,8-TriCDF		0.3	+ 10-fold
3,3′,4,4′,5-PentaCB	0.1	0.1	=
4′-B,3,3′,4,5-TetraCB		0.3	+ 3-fold
3′,4′-DiB,3,4,5-TriCB		0.1	=
3′,4′,5-TriB,3,4-DiCB		0.3	+ 3-fold
3,3′,4,4′,5-PentaBB		0.03	− 3-fold
2,3,3′,4,4′-PentaCB	0.00003	0.000003	− 10-fold
4′-B,2,3,3′,4-TetraCB		0.00001	− 3-fold
2,3′,4,4′,5-PentaCB	0.00003	0.000003	− 10-fold
4′-B,2,3′,4,5-TetraCB		0.00003	=
2,3,3′,4,4′,5-HexCB	0.00003	0.0003	+ 10-fold
4′-B,2,3,3′,4,5-PentaCB		0.0003	+ 10-fold

[a] TEFs from van den Berg et al., 2006.
[b] REP values from this study were rounded to the nearest half log unit to allow more simplistic comparison with the TEFs.

4.2. Antagonistic effects of mono-ortho-PCBs

Several mono-*ortho*-substituted PCBs and PXBs were tested in rat H4IIE cells and showed that all but PCB 156 were partial agonists. A concentration of 1 μM PXB 105 was shown to reduce the potency of TCDD by 200-fold showing that the compound is approximately a 20-fold more potent antagonist than PCB 105. A concentration of 300 nM PXB 118 was shown to reduce the potency of TCDD by 3-fold making it equivalent to PCB 118 in terms of antagonistic potential. PXB 156 was shown to be a potent antagonist with a concentration of 100 nM PXB 156 reducing the potency of TCDD by 100-fold, whereas PCB156 had

no detectable antagonistic activity. The substitution of chlorine for a bromine atom had no effect on the agonistic properties of the compound but it had a significant effect on the antagonistic potential.

In human MCF-7 cells, PCB 118 and PCB 156 were shown to be partial agonists whereas PCB 105 was shown to be a relatively potent antagonist. A concentration of 3 μM PCB 105 in rat and 10 μM PCB 105 in human reduced the potency of TCDD by ~30-fold. Further to this, a concentration of 3 μM PCB 118 in rat and 10 μM PCB 118 in human reduced the potency of TCDD by 10- to 25-fold. Binding data for these compounds is required to fully characterise their antagonistic properties. PCB 156 was shown to have very weak antagonistic properties in human and thus would have a very limited effect on the TEQ from an antagonism perspective.

Based on these data, a number of environmentally important PCBs have significant antagonistic activity in both rat and human cells. All of these compounds are present in the environment (Ahlborg et al., 1992; Fernandes et al., 2004, 2008, 2014; Larebeke et al., 2001; Kalantzi et al., 2004; Polder et al., 2008; Safe, 1990, 1994) and some make substantial contributions to the total measurement of TEQ. Therefore there might be significant consequences for the normal TEF-based method for deriving a TEQ, if the antagonistic effects of these compounds were taken into account. Thus, it is important to develop methodology to take these effects into account.

5. Conclusion

Most of the selected PXDDs tested in this study were found to have a slightly lower potency than their fully chlorinated counterparts whereas PXDFs and PXBs were shown to have a higher potency. It should however be noted that the compounds tested in this study are only a fraction of the total number of PXDD/Fs and PXBs therefore further testing would be required to fully qualify this observation. A new table of REPs has been produced for these compounds based on the data derived from this paper which reflect their potency more accurately than the current TEFs for chlorinated compounds. Recent research has shown the prevalence of these compounds demonstrating the importance of accurate potency estimation for appropriate TEQ calculation. Fernandes et al. (2014) recently reported the occurrence of PXDDs, PXDFs and PXBs, in comparison to the chlorinated congeners, in a wide variety of common food items and showed that in most cases the addition of mixed halogenated compounds to the TEQ significantly increased the contribution to dioxin-like toxicity.

This paper has also shown that PCB 105 and PCB 118 are partial agonists, although they are essentially antagonists at all but the highest of concentrations. The TEQ method assumes that all of the compounds in the mixture are pure agonists and thus does not account for the antagonistic properties of a compound. This study has demonstrated that some of the compounds included in the TEQ method are partial agonists and decrease the ability of TCDD to induce CYP1A1. Consideration should therefore be taken regarding the antagonistic properties of the PCBs and PXBs in relation to their ability to reduce the potency of other more potent AhR agonists.

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